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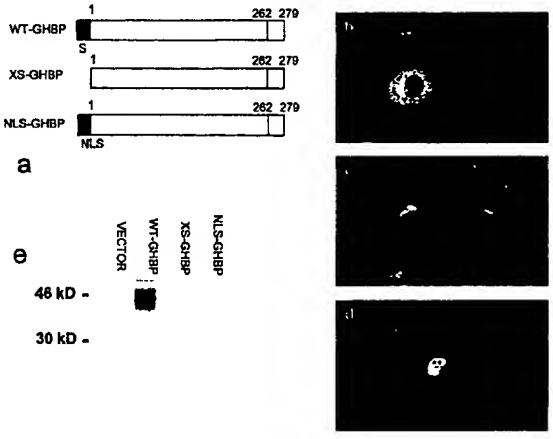

(57) Abstract

Constructs coding for NLS–GHBP are claimed. Also transgenic non–human animals expressing NLS–GHBP are claimed, and a method for producing such animals comprising introducing a NLS–GHBP expression plasmid into the pronucleus of a fertilised ovum, transferring the ovum to the reproduction tract of a recipient animal, letting the ovum develop, analysing the resulting offspring and identifying transgenic animals in which the NLS–GHBP expression plasmid have been integrated. Transgenic agricultural animals expressing NLS–GHBP produce more milk and/or meat than non–transgenic animals. The constructs and transgenic animals as well as tissue, cell cultures and derived from such animals are suitable models for the study of disorders affecting systems dependent on signal transduction through the JAK–STAT pathway, and also for screening compounds for treatment of such disorders. Substances that upon administration stimulate the interaction between GHBP and intracellular signalling molecules, simulate GHBP in interaction with intracellular signalling molecules, or lead to intracellular production of growth hormone binding protein are also claimed.

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USE OF GROWTH HORMONE BINDING PROTEIN WITH A NUCLEAR LOCALIZATION SEQUENCE (NLS-GHBP)

Technical field of the invention

The present invention relates to constructs coding for NLS-GHBP, transgenic non-human animals expressing NLS-GHBP, new model systems, and new pharmaceutical substances and preparations.

Background art

Growth hormone is a protein hormone found in man and other vertebrates. Growth hormone is the major regulator of postnatal body growth.

Growth hormone is thought to initiate its biological actions, including the induction of a number of RNA species in mammalian tissues, by interaction with a specific membrane bound receptor.

Several receptors belonging to the cytokine receptor superfamily, such as the GH receptor, exist in a soluble and transmembrane form. The functions of the transmembrane forms are well documented and include signal transduction through the JAK-STAT pathway resulting in gene transcription (JAK-STAT = janus kinase-signal transfer transducer activator of transcription, which is well-known to everybody skilled in the art). The role of the soluble receptors, with the notable exception of the IL-6 and CNTF soluble receptors, appears confined to ligand sequestration in the extracellular space with a consequent impairment of the cellular response to exogenous ligand.

A soluble rat growth hormone binding protein (GHBP) has been described that is derived from the GH receptor gene by an alternative mRNA splicing mechanism such that the transmembrane and intracellular domains of the GH receptor are replaced by a hydrophilic carboxyl terminus. In human and rabbit the GHBP is produced by proteolytic cleavage of the extracellular domain of the GH receptor.

In isolation, the GHBP is inactive although it does compete with the receptor for ligand binding in the extracellular space and therefore inhibits the cellular response to GH. The GHBP is located intracellularly and is translocated to the nucleus upon ligand stimulation. Other components of the GH signal transduction pathway are also located in the nucleus or translocate to the nucleus upon GH stimulation. Thus, the GH receptor is subject to ligand dependent nuclear translocation and constitutively nuclear JAK2 is phosphorylated by exogenous GH stimulation. Internalisation of the GH receptor has been reported not to be necessary to achieve transcriptional activation by GH and therefore the function of the nuclear localisation of components of the GH signal transduction pathway is unknown.

The inventors of the present invention have earlier provided a function for the nuclear localisation for some of the components of the GH signalling pathway. The nuclear localisation of the GHBP and the GH stimulated nuclear translocation of the alternatively spliced GHBP has been reported previously. The inventors of the present invention have also earlier reported that both GH and the GH receptor are subject to a rapid and transient nuclear translocation. At least one function of the nuclear translocation of the hormone and receptor appears to be the phosphorylation of nuclear localised JAK2. While a purely cytoplasmic transcriptional activation may be observed, the full transcriptional response requires nuclear localisation of at least several of the signalling components. Thus, physiological factors promoting the nuclear location of the GHBP would enhance the otherwise limited transcriptional response of the cell to various ligands.

Growth potentiating agents such as growth hormone have been used for widespread agricultural application to increase the product yield. Growth hormone (GH), also called somatotropin, is in some countries used to in-

crease meat or especially milk production from cattle. For example, if growth hormone is administered to a milk cow, the cow will produce 10-25% more milk than before treatment. Generally, the growth hormone used is bovine somatotropin, which must be administered by injection.

Also transgenic animals have been used, but this has been associated with several problems. One of the problems preventing the widespread commercial application of growth hormone to transgenic technology is the fact that GH transgenic animals suffer from glomerulosclerosis ultimately leading to chronic renal failure and premature death (see e.g. Pursel, V. G., et al., J. Anim. Sci. 71:10-17, 1993, and Doi, T., et al., Am. J. Pathol. 131:398-403, 1988). The precise mechanism by which this glomerulosclerosis occurs is not certain. Cells of the glomerulus do not express detectable levels of GH receptor and are therefore presumably incapable of a direct GH response.

Summary of the invention

One object according to the present invention is to provide a new NLS-GHBP encoding construct, the application of which will be further described below. Another object is to provide transgenic non-human animals that can be used for agricultural purposes, said animals lacking the disadvantages associated with the known GH transgenic agricultural animals.

Another object of the invention is to provide new model systems, which will make it possible to study disorders affecting systems in which STAT participates in the signalling, both in vivo and in vitro. Today, no adequate model systems exist.

Another object of the present invention is to provide substances and new pharmaceutical preparations which can be used for treatment of disorder caused by disturbances in the interaction between GHBP and intracellular signalling molecules.

Thus, the present invention relates to a construct coding for NLS-GHBP, which is a protein essentially consisting of a growth hormone binding protein (GHBP) in which the aminoterminal secretion sequence has been replaced by a nuclear localisation sequence (NLS).

The invention also relates to a transgenic non-human animal expressing NLS-GHBP.

Furthermore, the invention relates to a method for producing a transgenic non-human animal expressing NLS-GHBP said method comprising the following steps:

a) introducing a NLS-GHBP expression plasmid into the pronucleus of a fertilised ovum;

b) transferring the ovum to the reproduction tract of a recipient non-human animal and letting the ovum develop; and

c) analysing the off-spring resulting from step b) and identifying transgenic animals in which the NLS-GHBP expression plasmid has been integrated. The invention also relates to a transgenic non-human animal produced by the above-described method.

Moreover, the invention relates to tissue, cell cultures or cells derived from the above-mentioned transgenic non-human animal or from a transgenic non-human animal produced as described above.

The invention also relates to use of such constructs, animals, tissues, cell cultures or cells for the study of disorders in a system dependent on signal transduction through the JAK-STAT pathway, or for screening a compound for treatment of disorders in a system dependent on signal transduction through the JAK-STAT pathway.

The invention also relates to use of a low-molecular, plasma membrane permeable substance or a pharmaceutical preparation that upon administration to a patient will stimulate the interaction between GHBP and intracellular signalling molecules.

The invention also relates to use of a low-molecular, plasma membrane permeable substance or a pharmaceutical preparation that upon administration to a patient will simulate the GHBP in interaction with intracellular signalling molecules.

Finally, the invention relates to use of a low-molecular, plasma membrane permeable substance or a pharmaceutical preparation that upon administration to a patient will lead to intracellular production of growth hormone binding protein.

The characterising features or the invention will be evident from the following description and the appended claims.

Detailed description of the invention

The abbreviation NLS used herein stands for nuclear localisation sequence.

The abbreviation GHBP used herein stands for growth hormone binding protein.

The abbreviation rGHBP used herein stands for rat GHBP.

The abbreviation GH used herein stands for growth hormone (regardless of species origin).

The abbreviation hGH used herein stands for human GH.

The abbreviation EPO used herein stands for erythropoietin.

The abbreviation PRL used herein stands for prolactin.

During the work and research leading to the present invention it was shown that nuclear localised GHBP functions as a potent enhancer of STAT5 mediated transcription, not only for GH but also for other members of the cytokine receptor superfamily. Thus, the GHBP exerts opposing effects on STAT5 mediated transcription depending on its extra/intra-cellular location. STAT5 is a mammary

gland transcription factor known to persons skilled in the art.

The use of a soluble cytokine receptor as a location dependent transcriptional enhancer, and the ligand independent involvement of the extracellular domain of a polypeptide ligand receptor in intracellular signal transduction, provides additional novel mechanisms of transcriptional control.

Accordingly, it is shown herein that endogenously produced GHBP, in contrast to exogenous GHBP, is able to enhance the STAT5 mediated transcriptional response to GH. Interestingly, when the secretion sequence was removed, and the GHBP was targeted constitutively to the nucleus by addition of the nuclear localisation sequence of the SV40 large T antigen (NLS-GHBP), a further increase in transcriptional enhancement was obtained. The transcriptional enhancement did not require GH per se and was not specific to the GH receptor as similar enhancement of STAT5 mediated transcription by NLS-GHBP was obtained with specific ligand stimulation of both prolactin and erythropoietin receptors. Thus, the GHBP exerts divergent effects on STAT5 mediated transcription depending on its cellular location. The use of an alternatively transcribed cytokine receptor as a transcriptional enhancer to other cytokine receptor superfamily members provides an additional novel mechanism of transcriptional control.

It is thus possible to describe a new functional and ligand independent role for the soluble extracellular domains of cytokine receptors. The mechanism of transcriptional enhancement allows cross talk between receptor pathways utilising the same transcription factor. Thus, physiological factors that up-regulate the GHBP in a cell will increase the STAT5 mediated transcriptional responses to other ligands such as EPO. Non-GHBP binding ligands such as EPO would not be subject to the extracellular inhibition by GHBP, as would GH. It is possible

however, that EPO or other factors may modulate the secretion of the GHBP such that the response of the cell to GH is altered. Thus, the final hormonal response would depend on a complex interplay of the ratio of extracellular to intracellular (nuclear) GHBP and the identity of the stimulating ligand. Presumably the complexity of the response increases if other soluble cytokine receptors/binding proteins (such as PRL and EPO binding proteins) function as transcriptional enhancers like the GHBP. This regulatory strategy may also be one mechanism by which the cell can filter multiple redundant signals initiated by cytokine molecules sharing the same signal transduction pathway. Such regulatory mechanisms play an important role during physiological states such as puberty, pregnancy and lactation.

The use of a soluble cytokine receptor as a location dependent transcriptional enhancer, and the ligand independent involvement of the extracellular domain of a polypeptide ligand receptor in intracellular signal transduction, provides additional novel mechanisms of transcriptional control.

The NLS part of the protein resulting from the construct according to the invention may have the sequence of NLSs from many different sources. Any NLS be used to target the GHBP to the nucleus. More than 38 nuclear localisation sequences have been described in the literature (see e.g. Dingwall, C., et al., Nuclear targeting sequences - a consensus, Trends Biochem. Sci. 16:478-481, 1991, and Silver, P. A., How proteins enter the nucleus, Cell 64:489-497, 1991).

The use of one specific NLS is illustrated in the examples below. This is the NLS of the SV40 large T antigen, with the protein sequence:

M P K K K R K V.

Also the species origin of the GHBP part may vary, although rat GHBP is used in the examples below to illustrate the invention.

When NLS from the SV40 large T antigen and rat GHBP is used the construct according to the invention will have the sequence specified in SEQ ID NO: 1 in the sequence listing below. This is an example of a construct according to the invention. Other examples of the construct according to the invention are given in sequences with SEQ ID NOS 2-5 in the sequence listing below. It is also possible to use functionally equivalent homologues or analogues of those sequences.

There are two different ways the transgenic non-human animals according to the invention can express NLS-GHBP, either whole body expression or specific organ expression. The expression in specific organs is possible due to the fact that the protein is not secreted. Expression constructs for organ specific targeting of genes/cDNAs have been described earlier (regarding the mammary gland, see e.g. Uusi-Oukari, M., et al., Bovine alpha s 1 casein gene sequences direct high level expression of human granulocyte-macrophage colony stimulating factor in the milk of transgenic mice, Transgenic Res. 6:75-84, 1997; regarding muscle, see e.g. Sattler, W., et al., Muscle specific overexpression of lipoprotein lipase in transgenic mice results in increased alpha-tocopherol levels in skeletal muscle, Biochem. J. 318:15-19, 1996).

The transgenic non-human animals according to the invention may e.g. specifically express NLS-GHBP in the mammary gland or in muscles. This would result in anabolic changes in these organs that will lead to increased milk production or increased meat production, respectively. This is particularly useful in the agricultural fields.

These transgenic agricultural animals will set aside the need of GH administrations in order to increase milk or meat production, and they will alleviate the problems associated with the known transgenic animals used for those purposes. Transgenic animals expressing NLS-GHBP

have an increased responsiveness of the animal to its own endogenously produced GH, which means that the natural production of hormone in the transgenic animals will suffice for stimulation of NLS-GHBP, and it is thus possible to circumvent the problem associated with administration of GH leading to large amounts of circulating GH since the entire protein is expressed intracellular.

It is also possible to use the NLS-GHBP encoding constructs and the transgenic non-human animals according to the invention as model systems. Suitable animals for those purposes are rodents, such as rats or mice. It is also possible to use tissues, cell cultures or cells derived from a transgenic animal according to the invention as model systems or for enhances cellular function such as in pharmaceutical production.

The above mentioned model systems are e.g. suitable for the study of disorders in a system dependent on signal transduction through the JAK-STAT pathway, such as the growth hormone system, the prolactin system, the erythropoietin system and the interleukin system.

The model systems can also be used for screening compounds for treatment of disorders in a system dependent on signal transduction through the JAK-STAT pathway, such as the growth hormone system, the prolactin, the erythropoietin system and the interleukin system.

The present invention also relates to the use of a low-molecular, plasma membrane permeable substance that upon administration to a patient will stimulate the interaction between GHBP and intracellular signalling molecules, or simulate the GHBP in interaction with intracellular signalling molecules, or lead to intracellular production of growth hormone binding protein, which will increase the effect of endogenous growth hormone, prolactin and erythropoietin.

The invention also relates to pharmaceutical preparations comprising such substances and to the

production of pharmaceutical preparations by use of such substances.

The above mentioned substances and pharmaceutical preparations can be used for treatment of diseases such as dwarfism, osteoporosis, hepatic failure, atrophic skin diseases, immunodeficiency since the stimulation may result in enhancement of the growth hormone system resulting in increased growth of bone, heart, skin, liver, cells of the immune system.

The stimulation can also be exerted in other organs resulting in alteration of the metabolism or behavioural effects and the above mentioned substance and pharmaceutical preparation can thus be used for treatment of diseases such as atherosclerosis, coronary heart disease, stroke, depression or affective psychiatric diseases.

Furthermore, the stimulation can enhance the erythropoietin system resulting in increased haematopoiesis, above mentioned substance and pharmaceutical preparation treatment of anaemia.

Moreover, the stimulation can result in enhancement of the prolactin system stimulating milk production or immune effects, and the above mentioned substance and pharmaceutical preparation can thus be used for treatment of lactation disturbances or immunodeficiency.

Finally, the stimulation can also affect other receptors in the cytokine receptor superfamily, including, but not excluding others, interleukin receptors. This results in stimulation of the immune system, and the above mentioned substance and pharmaceutical preparation can thus be used for treatment of immunodeficiency.

The invention will now be further explained in the examples below. The examples are only intended to illustrate the invention and should in no way be considered to limit the scope of the invention.

Brief description of the drawings

In the examples below, references is made to the accompanying drawings on which:

Fig. 1 illustrates the effect of exogenous recombinant rat GHBP on GH induction of STAT5 mediated transcription in BRL-GHR_{1-638} cells;

Fig. 2 a is a schematic diagram of the WT-GHBP, XS-GHBP and NLS-GHBP proteins encoded by their respective cDNAs;

b illustrates immunofluorescent localisation of the expressed proteins in BRL cells for WT-GHBP expressed in the cytoplasm;

c illustrates immunofluorescent localisation of the expressed proteins in BRL cells for WT-GHBP expressed in the perinuclear region of the cell;

d illustrates immunofluorescent localisation of the expressed proteins in BRL cells for NLS-GHBP expressed in the nucleus;

e illustrates Western blot analysis of media from BRL cells transiently transfected with WT-GHBP, XS-GHBP or NLS-GHBP cDNAs;

Fig. 3 a illustrates the effect of increasing concentrations of hGH on the transcriptional response to hGH in the presence of transiently transfected vector and WT-GHBP cDNA;

b illustrates the effect of transient transfection of XS-GHBP cDNA on the transcriptional response to hGH in BRL cells transiently transfected with GH receptor cDNA;

Fig. 4 a illustrates the effect of transient transfection of NLS-GHBP cDNA on the transcriptional response to hGH in BRL cells transiently transfected with GH receptor cDNA;

b illustrates the effect of increasing concentrations of hGH on the transcriptional response to hGH in the presence of transiently transfected

vector and NLS-GHBP cDNA;

c illustrates the effect of increasing concentrations of rGH on the transcriptional response to hGH in the presence of transiently transfected vector and NLS-GHBP cDNA;

Fig. 5 illustrates the effect of transient transfection of NLS-GHBP cDNA on the transcriptional response to hGH and rGH, oPRL and mEPO in BRL cells transiently transfected with the GH receptor, PRL receptor or EPO receptor cDNAs respectively.

Examples

The BRL (Buffalo rat liver) cell co-transfection assay described by Sliva, D., et al., J. Biol. Chem., 261:26208-26214, 1994 was used to study the role of the GHBP in the signal transduction pathway of GH.

The human growth hormone used was a gift from Novo Nordisk (Singapore) and Pharmacia-Upjohn (Stockholm, Sweden). All cell culture medium and the supplements for culture medium were obtained from Sigma (St. Louis, MO). The luciferase assay system was purchased from Promega (Madison, WI). The ECL kit was obtained from Amersham (UK). The GH, PRL and EPO receptor cDNAs used here have all been described previously (Wood, T. J. J., et al., Mol. Cell. Endo., 130:69-81, 1997.

Generation of stable cell transfectants:

Buffalo rat liver (BRL) cells were stably transfected with the complete rat GH receptor cDNA inserted into an expression vector containing the human cytomegalovirus enhancer and promoter (pcDNA1). The characterisation and use of these cells has previously been described in detail by Sliva, D., et al., in J. Biol. Chem. 269:26208-26214, 1994. These cells will below be referred to as BRL-GHR_{1-638} cells.

Cell culture:

BRL cells were grown in Dulbecco's Modification of Eagles Medium (DMEM) supplemented with 10 % heat inactivated foetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine, at 37°C in 5% CO_2. Human growth hormone (hGH), rat growth hormone (rGH), ovine prolactin (oPRL) and recombinant rat GHBP were prepared as a stock solution of I mg/n-A in distilled water. For treatment of cells, hGH, rGH, oPRL, mouse erythropoietin (mEPO) and recombinant rat GHBP were diluted in fresh DMEM serum free medium and added to the cells after transient transfection. Cells were treated with 100 nM hGH unless otherwise specified. oPRL was used at 100 nM. mEPO was used at 10 U/ml.

Construction of GHBP expression plasmids:

The cDNA expression plasmid encoding the wild type GHBP under the control of the metallothionein Ia promotor was as previously described by Möller, C., et al., in J. Biol. Chem. 267:23403-23408, 1992. In the XS-GHBP construct, the rat GHBP was PCR amplified without its signalling peptide and an ATG was introduced in the primer just upstream of where the mature GHBP protein is coded. The NLS-GHBP was constructed in a similar way but a nuclear localisation signal from the SV40 Large T antigen (with the protein sequence P K K K R K V) was added upstream of where the mature GHBP is coded. The integrity of the reading frame for the GHBP modifications were confirmed by sequence analysis.

Transient Transfection and Reporter Assay:

BRL and BRL-GHR_{1-638} cells were cultured to confluence in six well plates. Transient transfection was performed in serum free DMEM with DOTAP (a well-known transfection reagent) according to the manufacturers instructions. 1 µg of reporter plasmid (SPI-GLE1-CAT) and 1 µg of pSV2-LUC were transfected per well. The control or empty vec-

tors served to normalise the amount of DNA transfected. For receptor cDNA transfection into BRL cells 1 µg of each receptor cDNA was used. Cells were incubated with DOTAP/DNA for 12 hours before the media was changed to serum free DMEM containing either the respective hormones or GHBP at the indicated concentrations. After a further 24 hours, cells were washed in PBS and scraped into lysis buffer. The protein content of the samples were normalised and CAT and luciferase assays were performed as previously described by Wood, T. J. J., et al., in J. Biol. Chem. 270:9448-53, 1995. The results were normalised to the level of luciferase to control for transfection efficiency and calculated as the fold stimulation of unstimulated (non-hormone treated) cells.

Light microscopic immunocytochemistry:

BRL cells were grown on glass coverslips in six well plates and transiently transfected as described above. Fixation was performed with PBS pH 7.4 containing 4 % paraformaldehyde for 10 minutes at room temperature. Cells were permeabilised with PBS- 0.1% Triton X-100 for 1 minute and processed for immunocytochemistry as described by Lobie, P. E., et al., in J. Biol. Chem. 269:31375-31746, 1994. The location of the expressed GHBP was determined using the Mab 4.3 directed against the hydrophilic carboxylterminus of the GHBP as described by Lobie, P. E., et al., in Endocrinology 130:3057-3065, 1992. Non-cross reactive Mabs 50.8 and 7 at the same were used as control.

Immunoprecipitation and Western Blot Analysis:

Media from BRL cells transiently transfected with the different GHBP constructs was collected and concentrated. The fractions were normalised for protein content and loaded onto a 7.5 % polyacrylamide gel as described by Lobie, P. E., et al., in J. Biol. Chem. 269:31375-31746, 1994. The proteins were transferred to

nitrocellulose membranes using a semi-dry apparatus in Laemlli electrophoresis buffer containing 15% methanol. The membranes were blocked for 1 hour with 5% skim milk powder in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.4). Mab 4.3 at 0.25 µg/ml in TTBS (TBS plus 0.1% Tween 20) with 1% skim milk powder was used for GHBP detection. The membranes were further processed and developed using the ECL system as previously described by Lobie, P. E., et al., in J. Biol. Chem. 269:31375-31746, 1994.

Example 1

The BRL cell line stably transfected with GH receptor cDNA (BRL-GHR_{1-638}) as described above was used to demonstrate the effect of exogenous rat GHBP on GH stimulation of CAT production from the STAT5 responsive region of the serine protease inhibitor 2.1 gene promoter (SPI-GLE1-CAT) (which has been described by Wood, T. J. J., et al. in J. Biol. Chem. 270:9448-53, 1995). Exogenously added recombinant rat GHBP decreased in a dose dependent manner the hGH stimulation of SPI-GLE1-CAT, which is illustrated in figure 1. The BRL-GHR_{1-638} cells were cultured to confluence and transiently transfected with SPI-GLE1-CAT as described as described above. The cells were treated for 24 hours with 1 nM hGH in the presence of recombinant rat GHBP with the concentrations indicated in the figure. The results are presented as the mean ± SD of triplicate determinations of the fold stimulation above non-hormone stimulated cells.

The results show that exogenously applied GHBP functioned as expected (e.g. according to Lim, L., et al., Endocrinology 127:1287-1291, 1990) and reduced the cellular response to GH.

Example 2

In this example the effects of endogenously produced GHBP on hGH transcriptional activation was studied. For these experiments BRL cells of the wild type-GHBP (WT-

GHBP), a GHBP with the aminoterminal secretion sequence removed (XS-GHBP) and a GHBP with the aminoterminal secretion sequence replaced by the nuclear location sequence of SV40 large T antigen (NLS-GHBP) was used. Schematic diagrams of the WT-GHBP, XS-GHBP and NLS-GHBP proteins encoded by their respective cDNAs are shown in figure 2 a. The BRL cells were transiently transfected with rat GH receptor cDNA, WT-GHBP cDNA (Möller, C., et al., J. Biol. Chem. 267:23403-23408, 1992) and SPI-GLE1-CAT (Sliva, D., et al., J. Biol. Chem. 269:26208-26214, 1994) as described above.

The immunofluorescent localisation of the expressed proteins in BRL cells was determined through detection by use of Mab 4.3 directed against the hydrophilic carboxyl-terminus of the GHBP. WT-GHBP was expressed in the cytoplasm, as shown in figure 2 b, XS-GHBP was expressed in the perinuclear region of the cell, as shown in figure 2 c, and NLS-GHBP was expressed in the nucleus, as shown in figure 2 d.

Finally a Western blot analysis was made of media from BRL cells transiently transfected with WT-GHBP, XS-GHBP or NLS-GHBP cDNAs. The result is shown in figure 2 e.

Example 3

In contrast to exogenously added GHBP, 1 µg of transiently transfected WT-GHBP cDNA resulted in a significant increase in the STAT5 mediated transcriptional response to GH. The effect of increasing concentrations of hGH on the transcriptional response to hGH in the presence of transiently transfected vector and WT-GHBP cDNA was then studied. BRL cells were cultured to confluence and transiently transfected with GH receptor cDNA, SPI-GLE1-CAT and 1 µg WT-GHBP cDNA as described above. The cells were treated for 24 hours with hGH in the concentrations indicated in figure 3 a. The results are presented in the figure as the mean ± SD of triplicate de-

terminations of the fold stimulation above non-hormone stimulated cells.

Example 4

Thereafter the effect of transient transfection of XS-GHBP cDNA on the transcriptional response to hGH in BRL cells transiently transfected with GH receptor cDNA was studied. BRL cells were cultured to confluence and transiently transfected as described above with GH receptor cDNA, SPI-GLE1-CAT and GHBP cDNA in the amounts indicated in figure 3 b. The cells were treated for 24 hours with 100 nM hGH. The results are presented in figure 3 b as the mean ± SD of triplicate determinations of the fold stimulation above non-hormone stimulated cells.

The transfection of increasing amounts of WT-GHBP cDNA resulted in less enhancement of the hGH transcriptional response presumably due to increased secretion of GHBP to the media. WT-GHBP exerted no effect on transcription in the absence of receptor. The increase in GH stimulated transcription was evidenced at even the lowest hGH concentration thereby suggestive that enhancement of transcription was not a simple ligand sequestration effect, as is shown in figure 3. These results suggested that extracellular and intracellular GHBP exerted opposing effects on GH stimulated transcription.

To test this hypothesis the secretion sequence from the GHBP cDNA (XS-GHBP) (e.g. described by Baumbach, W. R., et al., Genes Dev. 3:1199-1205, 1989) was removed. Thus XS-GHBP was confined to the perinuclear cytoplasm of the cell and was not secreted to the media, as described above and as illustrated in figure 2 c. Transient transfection of receptor and XS-GHBP cDNA again increased the transcriptional response to GH but only slightly more than the secreted WT-GHBP when compared under the same conditions. Since the cytoplasmic localisation of the GHBP conferred transcriptional enhancement, the STAT5 mediated transcriptional response to GH in the presence of

GHBP artificially targeted to the nucleus was examined. The nuclear localisation of the GHBP (Lobie, P. E., et al., Endocrinology 130:3057-3065, 1992) and the GH stimulated nuclear translocation of the alternatively spliced GHBP (Goodman, H. M., et al., Proc. Endo. Soc. USA 76:928, 1994) has been reported previously. It has also been reported that both GH (Sliva, D., et al., J. Biol. Chem., 261:26208-26214, 1994) and the GH receptor (Lobie, P. E., et al., J. Biol. Chem. 266:22645-22652, 1991) are subject to a rapid and transient nuclear translocation. At least one function of the nuclear translocation of the hormone and receptor appears to be the phosphorylation of nuclear localised JAK2 (Lobie, P. E., et al., Endocrinology 137:4037-4045, 1996). The NLS of the SV40 large T antigen (with the sequence P K K K R K V)(e.g. described by Dingwall, C., et al., TIBS 16:478-481, 1991) was therefore introduced at the NH_2 terminal of the GHBP lacking the secretion sequence (NLS-GHBP). Thus, it was shown that NLS-GHBP was localised to the nucleus and was not secreted to the media, see figure 2 d.

Thereafter the effect of transient transfection of NLS-GHBP cDNA on the transcriptional response to hGH in BRL cells transiently transfected with GH receptor cDNA. BRL cells were cultured to confluence and transiently transfected with GH receptor cDNA, SPI-GLE1-CAT and the indicated amounts of NLS-GHBP cDNA as described above. The cells were treated for 24 hours with 100 nM hGH. The results presented in figure 4 a are presented as the mean ± SD of triplicate determinations of the fold stimulation above non-hormone stimulated cells. Thus, transient transfection of GH receptor cDNA with the cDNA for NLS-GHBP resulted in a marked transcriptional enhancement through SPI-GLE1-CAT. The transcriptional enhancing effect was increased with transfection of larger amounts of NLS-GHBP cDNA.

The effect of increasing concentrations of hGH and rGH, respectively on the transcriptional response to hGH

in the presence of transiently transfected vector and NLS-GHBP cDNA was also studied. BRL cells were cultured to confluence and transiently transfected with GH receptor cDNA, SPI-GLE1-CAT and 5 µg NLS-GHBP cDNA as described above. The cells were treated for 24 hours with hGH and rGH in concentrations shown in figure 4 b and 4 c, respectively. The results are presented in the figures as the mean ± SD of triplicate determinations of the fold stimulation above non-hormone stimulated cells. The transcriptional enhancing activity of NLS-GHBP was not observed when a c-fos (STAT1 and STAT3) reporter plasmid (Chen, C., et al., Endocrinology 136:4505-4516, 1995) was used and therefore appeared specific for STAT5 mediated responses (data not shown). No transcriptional response to GH was obtained upon transfection of NLS-GHBP cDNA without GH receptor cDNA. BRL cells stably transfected with NLS-GHBP cDNA and transiently transfected with GH receptor cDNA also displayed enhanced GH dependent CAT activity compared to vector transfected control cells.

Example 5

Finally it was examined if NLS-GHBP could function as a transcriptional enhancer for other cytokine receptor superfamily members that also utilise STAT5 for transcriptional activation (Gouilleux, F., et al., EMBO J. 2005-2013, 1995). The effect of transient transfection of NLS-GHBP cDNA on the transcriptional response to hGH and rGH, oPRL and mEPO in BRL cells was studied. The BRL cells were cultured to confluency and transiently transfected with either GH receptor, PRL receptor or EPO receptor cDNAs, SPI-GLE1-CAT and 5 µg NLS-GHBP cDNA as described above. The cells were treated for 24 hours with 100 nM hGH or rGH, 100 mM oPRL or 10 U/ml mEPO respectively. The results are presented in figure 5 as the mean ± SD of triplicate determinations of the fold stimulation above non-hormone stimulated cells. Human GH is also a ligand for the PRL receptor (which has been described

e.g. by Wood, T. J. J., et al., Mol. Cell. Endo., 130:69-81, 1997) and therefore a transcriptional to hGH via the PRL receptor can be expected. The STAT5 mediated transcription, induced specifically through the PRL (with oPRL) or EPO receptors, was also enhanced in the presence of NLS-GHBP to a similar extent as the enhancement observed through the GH receptor. An expression plasmid encoding for NLS-hGH did not result in an EPO induced transcriptional enhancement through the EPO receptor and therefore the transcription enhancement of NLS-GHBP are not due to a general effect of the NLS of the SV40 large T antigen.

Examples 6-11 - construction of plasmids

In these examples six different NLS-GHBP expression plasmids were made using the same backbone-plasmid and into it ligating different variations of GHBP DNA. The GHBP DNA variants were made by the polymerase chain reaction (PCR) technique by amplifying rat GHBP complementary DNA (rGHBP cDNA) in all occasions as the template, if nothing else is specified below.

To make the different variations, different primers (one 3' primer and one 5' primer) were used in the PCR reaction.

After the PCR reaction the product was cut by NOT-1, gel cleaned, and ligated into the NOT-1 site.

Backbone plasmid

In the pUC BM20 plasmid the Acc I-Nar I was deleted in order to take polylinker sequence away. The mouse-metallotionein 1 promoter from Kpn I to BamH I followed by the hGH gene from BamH I to EcoR I was inserted. The ATG of the hGH was mutated into a TTG. The EcoN I site in exon 2 of hGH was converted into a Not I site. The plasmid was opened in this site for ligation of the different construct-sequences of GHBP.

Example 6 - NLS-rGHBP 1- 279 (full length) construct PCR

The 5′ primer: (GAC CGA TAT CGA GCG GCC GCC TAG CTG CAA TGC CAA AAA AGA AGA GAA AGG TAA CAC CAG CTA CTC TTG GC) consist of restriction enzyme NOT-1 recognition sequence, the KOZAK sequence from the hGH gene, the sequence encoding the nuclear transport peptide (NLS) (aminoacids M, P, K, K, K, R, K, V) followed by sequence complementary to the first 18 bases in the rGHBP gene.

The 3′ primer: (ACT AAT GCG GCC GCA GGG ATG GCA GAT CCT CT) has sequence complementary to the last 18 bases in rGHBP, followed by NOT-1 recognition sequence.

Product: This construct produces the full-length ratGHBP (aminoacids 1-279) with the nuclear transport peptide in front (see SEQ ID NO 1 in the sequence listing below).

Example 7 - NLS-rGHBP 1-262 (no tail) construct PCR

The 5′ primer: the same 5′ primer as in example 6 was used.

The 3′ primer: (CCA CTC TGA ATG CGG CCG CTC AGG CTA GTT ATT CTT CAC ATG CTG CCA GT) has sequence complementary to bases encoding the aminoacids 257-262 in rGHBP followed by stop codons in three different reading frames and NOT-1 recognition sequence.

Product: This construct produces rGHBP without the hydrophilic tail (aminoacids 1-262) with the nuclear transport peptide in front (see SEQ ID NO 2 in the sequence listing below).

Example 8 - NLS-rGHBP 1-115 construct PCR

The 5′ primer: The same 5′ primer as in example 6 was used.

The 3′ primer: (CCA CTC TGA ATG CGG CCG CTC AGG CTA GTT AGA AGT AAC AGC TGT TTG CTC CAG CAG A) has sequence complementary to bases encoding the aminoacids 107-115 in

rGHBP followed by stop codons in three different reading frames and NOT-1 recognition sequence.

Product: This construct produces a truncated rGHBP (aminoacids 1-115) with the nuclear transport peptide in front (see SEQ ID NO 3 in the sequence listing below).

Example 9 - NLS-rGHBP 1-115 mutated 104 construct PCR:

The 5′ primer: The same 5′ primer as in example 6 was used.

The 3′ primer: (CCA CTC TGA ATG CGG CCG CTC AGG CTA GTT AGA AGT AAC AGC TGT TTG CTC CAG CAG AGA CAA AAT CAG GGC ATT) has sequence complementary to bases encoding the aminoacids 102-115 in rGHBP with a point-mutation at the aminoacid in position 104 followed by stop codons in three different reading frames and NOT-1 recognition sequence.

Product: This construct produces a truncated rGHBP (aminoacids 1-115) with a point-mutation which will give rise to a phenylalanine at the aminoacid in position 104 instead of a tyrosine and the nuclear transport peptide in front (see SEQ ID NO 4 in the sequence listing below).

Example 10 - rGHBP 1-279 construct PCR

The 5′ primer: (GAC CGA TAT CGA GCG GCC GCC TAG CTG CAA TGA CAC CAG CTA CTC TTG GC) consist of restriction enzyme NOT-1 recognition sequence, the KOZAK sequence from the hGH gene, followed by sequence complementary to the first 18 bases in the rGHBP gene.

The 3′ primer: The same 3′ primer as in example 6 was used.

Product: This construct produces the full-length rGHBP (aminoacids 1-279) without any nuclear transport peptide.

Example 11 - NLS-rGHBP 1-279 mutated 104 construct PCR

This construct was made by the running of 3 PCRs after each other.

Reaction A:

The 5′ primer: The same 5′ primer as in example 6 was used.

The 3′ primer: (TGC TCC AGC AGA GAC AAA ATC AGG GCA TTC T) consist of 31 bases complementary to rGHBP but with a point-mutation at the aminoacid in position 104.

Reaction B:

The 5′ primer: (TGC CCT GAT TTT GTC TCT GC) consist of 20 bases complementary to rGHBP but with a point-mutation at the aminoacid in position 104.

The 3′ primer: The same 3′ primer as in example 6 was used.

Reaction C:

The products from reaction A and B was cleaned on gel and mixed in equal proportions and then used as template for reaction C.

The 5′ primer: The same 5′ primer as in example 6 was used.

The 3′ primer: The same 3′ primer as in example 6 was used.

Product: This construct produces the full-length rGHBP (aminoacids 1-279) with a point-mutation which will give rise to a phenylalanine at the aminoacid in position 104 instead of a tyrosine and with the nuclear transport peptide in front (see SEQ ID NO 5 in the sequence listing below).

Example 12 - production of transgenic mice

Transgenic mice were developed by microinjection of DNA into pronuclei of fertilised oocytes obtained from C57BlxCBA F1 female mice after superovulation with 5 IU human chorionic gonadotropin followed by 5 IU pregnant mare´s serum gonadotropin 48 h later. The females had been mated to C57BlxCBA male mice. The injections were performed using a Nikon inverted microscope equipped with Nomarski optics and Narishigi micromanipulators. A EcoRI-Asp718 fragment from the plasmid NLS-GHBP described

in example 6, i.e. (see SEQ ID NO 1 in the sequence listing below) (which contains the full-length rGHBP sequence (aminoacids 1-279) with the nuclear transport peptide in front, regulated by the Mt promotor ligated into exon 2 of the inactivated hGH gene) was isolated using agarose gel separation followed by isotachyphoresis. The DNA fragment was injected into the pronuclei in a concentration of 5-10 ng/µl. 126 injected oocytes were implanted into 8 pseudopregnant C57BlxCBA F1 foster mice obtained after mating with vasectomised male C57BlxCBA mice. 18 mice were born and 0.5 cm biopsies from tails were taken from the mice at three weeks of age. DNA was isolated from the tail specimens after a 55°C overnight digestion in 0.5 ml of 50 mM Tris (pH 8), 100 mM EDTA, 0.5 % SDS and 25 µl proteinas K (10 mg/µl) solution followed by phenol and phenol/chloroform extractions. The DNA was precipitated by 50 µl sodium acetate (pH 6) and 0.5 ml 100% ethanol followed by washing in 70 % ethanol and then dissolved in 100 µl 10 mM Tris (pH 8), 1 nM EDTA.

The DNA was restricted using Sac-I and analysed by Southern blot analysis. A Nsi I-Hind III fragment from GHBP was labelled using random primer labelling and used as a probe. The animals were identified as transgenic.

SEQUENCE LISTING

<110> Isaksson, Olle
Toernell, Jan
Sandstedt, Jonas
Lobie, Peter Edward
Graichen, Ralph Eberhard
Institute of molecular and cell biology

<120> Use of growth hormone binding protein (GHBP)

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CLAIMS

1. A construct coding for NLS-GHBP, which is a protein essentially consisting of a growth hormone binding protein (GHBP) in which the aminoterminal secretion sequence has been replaced by a nuclear localisation sequence (NLS).

2. A construct according to claim 1, wherein the NLS part of the resulting protein resembles the NLS of SV40 large T antigen.

3. A construct according to claim 1 or claim 2, wherein the GHBP part of the resulting protein resembles rat GHBP.

4. A construct according to claim 2 and claim 3, with a sequence selected from the group consisting of the sequences with SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO4, and SEQ ID NO 5 given in the sequence listing, or functionally equivalent homologues or analogues thereof.

5. A transgenic non-human animal expressing NLS-GHBP.

6. A transgenic non-human animal according to claim 5 expressing NLS-GHBP in a specific organ.

7. An animal according to claim 6, wherein the organ is the mammary gland.

8. An animal according to claim 6, wherein the organ is a muscle.

9. A method for producing a transgenic non-human animal expressing NLS-GHBP said method comprising the following steps:

a) introducing a NLS-GHBP expression plasmid into the pronucleus of a fertilised ovum;

b) transferring the ovum to the reproduction tract of a recipient non-human animal and letting the ovum develop; and

c) analysing the off-spring resulting from step b) and identifying transgenic animals in which the NLS-GHBP expression plasmid have been integrated.

10. A method according to claim 9 wherein the NLS originates from the SV40 large T antigen.

11. A transgenic non-human animal produced by the method according to claim 9 or claim 10.

12. A transgenic non-human animal that is a descendent of an animal according to claim 11.

13. An animal according to any one of the claims 5-8, 11 or 12 wherein the animal is a mammal.

14. An animal according to claim 13, wherein the animal is an agricultural animal.

15. An animal according to claim 14, usable for milk production.

16. An animal according to claim 14, usable for meat production.

17. An animal according to claim 13, wherein the animal is a rodent.

18. An animal according to claim 17, wherein the animal is a rat.

19. An animal according to claim 17, wherein the animal is a mouse.

20. A tissue derived from an animal according to anyone of the claims 5-8 or 11-19.

21. A cell culture derived from an animal according to any of the claims 5-8 or 11-19.

22. A cell derived from an animal according to any of the claims 5-8 or 11-19.

23. Use of a construct according to any one of the claims 1-4, an animal according to any one of the claims 5-8 or 11-19, a tissue according to claim 20, a cell culture according to claim 21, or a cell according to claim 22 for the study of disorders in a system dependent on signal transduction through the JAK-STAT pathway.

24. Use of a construct according to any one of the claims 1-4, an animal according to any one of the claims 5-8 or 11-19, a tissue according to claim 20, a cell culture according to claim 21, or a cell according to claim 22 for screening a compound for treatment of

disorders in a system dependent on signal transduction through the JAK-STAT pathway.

25. Use according to claim 23 or 24, wherein the system dependent on signal transduction through the JAK-STAT pathway is the growth hormone system, the prolactin system or the erythropoietin system.

26. Use of a low-molecular, plasma membrane permeable substance that upon administration to a patient will stimulate the interaction between GHBP and intracellular signalling molecules.

27. Use of a low-molecular, plasma membrane permeable substance that upon administration to a patient will simulate the GHBP in interaction with intracellular signalling molecules.

28. Use of a low-molecular, plasma membrane permeable substance that upon administration to a patient will lead to intracellular production of growth hormone binding protein.

29. Use according to any one of the claims 26-28 for treatment of dwarfism, osteoporosis, hepatic failure, atrophic skin diseases, and immunodeficiency.

30. Use according to any one of the claims 26-28 for treatment of diseases, such as atherosclerosis, coronary heart disease, stroke, depression or affective psychiatric diseases.

31. Use according to any one of the claims 26-28 for treatment of anaemia.

32. Use according to any one of the claims 26-28 for treatment of lactation disturbances.

33. Use according to any one of the claims 26-28 for treatment of immunodeficiency.

34. A pharmaceutical preparation comprising an effective amount of a low-molecular, plasma membrane permeable substance that upon administration to a patient will stimulate the interaction between GHBP and intracellular signalling molecules.

35. A pharmaceutical preparation comprising an effective amount of a low-molecular, plasma membrane permeable substance that upon administration to a patient will simulate the GHBP in interaction with intracellular signalling molecules.

36. A pharmaceutical preparation comprising an effective amount of a low-molecular, plasma membrane permeable substance that upon administration to a patient will lead to intracellular production of growth hormone binding protein.

37. A pharmaceutical preparation according to any one of the claims 34-36 for treatment of dwarfism, osteoporosis, hepatic failure, atrophic skin diseases, immunodeficiency.

38. A pharmaceutical preparation according to any one of the claims 34-36 for treatment of diseases atherosclerosis, coronary heart disease, stroke, depression or affective psychiatric diseases.

39. A pharmaceutical preparation according any one of the claims 34-36 for treatment of anaemia.

40. A pharmaceutical preparation according to any one of the claims 34-36 for treatment of lactation disturbances.

41. A pharmaceutical preparation according to any one of the claims 34-36 for treatment of immunodeficiency.

42. Use of a low-molecular, plasma membrane permeable substance for the production of a pharmaceutical preparation which upon administration to a patient will stimulate the interaction between GHBP and intracellular signalling molecules.

43. Use of a low-molecular, plasma membrane permeable substance for the production of a pharmaceutical preparation which upon administration to a patient will simulate the GHBP in interaction with intracellular signalling molecules.

44. Use of a low-molecular, plasma membrane permeable substance for the production of a pharmaceutical preparation which upon administration to a patient will lead to intracellular production of growth hormone binding protein.

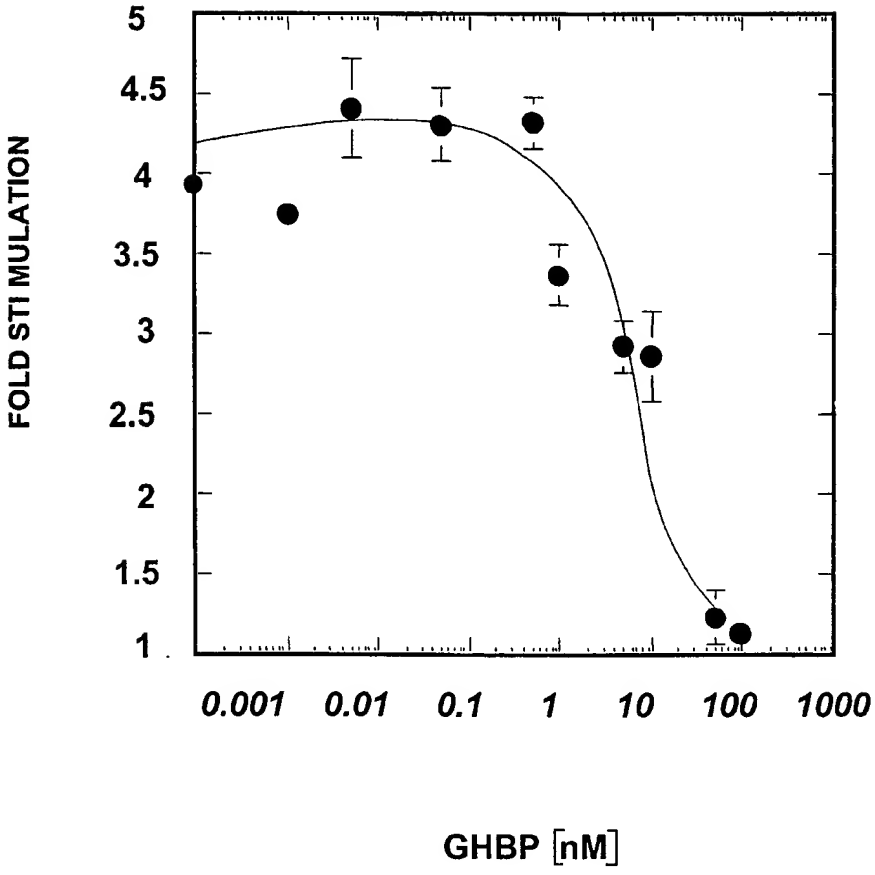
45. Use according to any one of the claims 42-44, said pharmaceutical preparation being intended for treatment of dwarfism, osteoporosis, hepatic failure, atrophic skin diseases, immunodeficiency.

46. Use according to any one of the claims 42-44, said pharmaceutical preparation being intended for treatment of diseases atherosclerosis, coronary heart disease, stroke, depression or affective psychiatric diseases.

47. Use according to any one of the claims 42-44, said pharmaceutical preparation being intended for treatment of anaemia.

48. Use according to any one of the claims 42-44, said pharmaceutical preparation being intended for treatment of lactation disturbances.

49. Use according to any one of the claims 42-44, said pharmaceutical preparation being intended for treatment of immunodeficiency.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4
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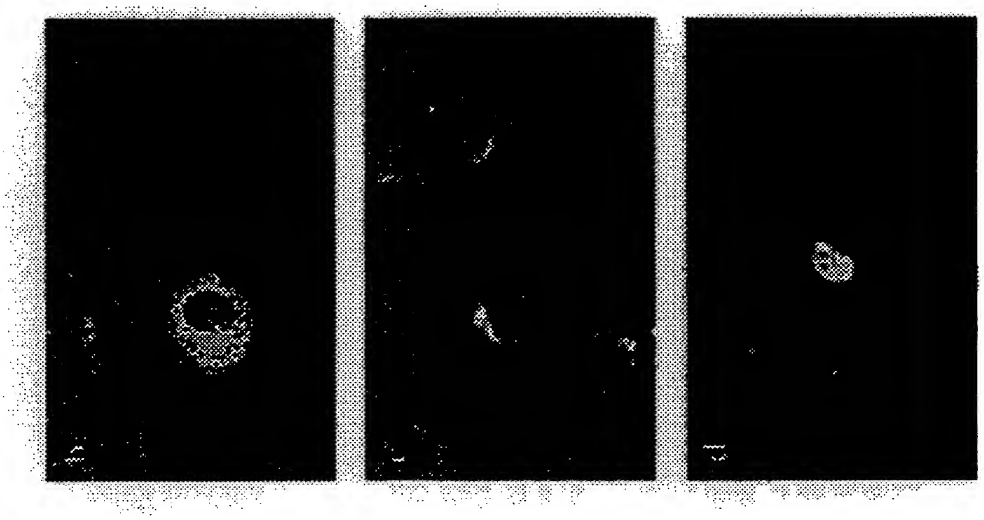
GHBP [nM]

Fig. 1



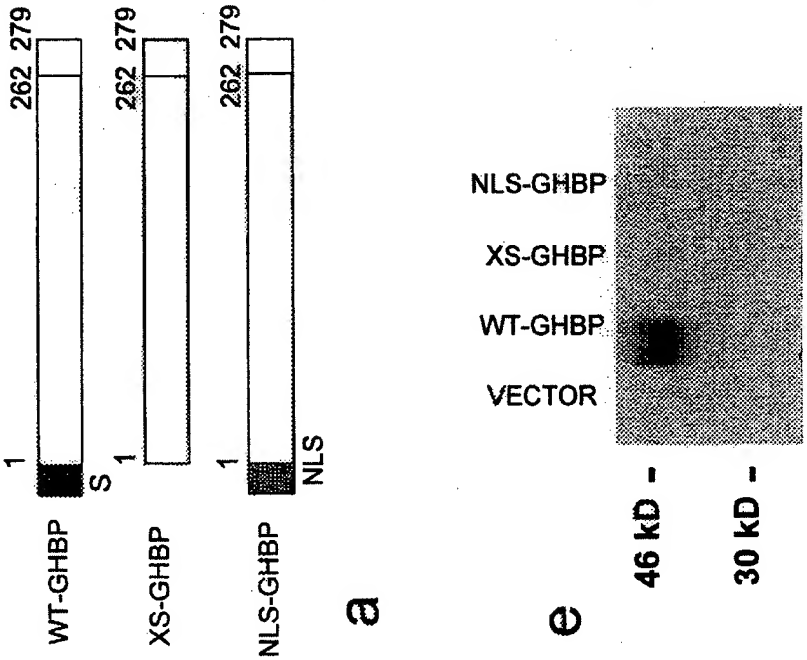

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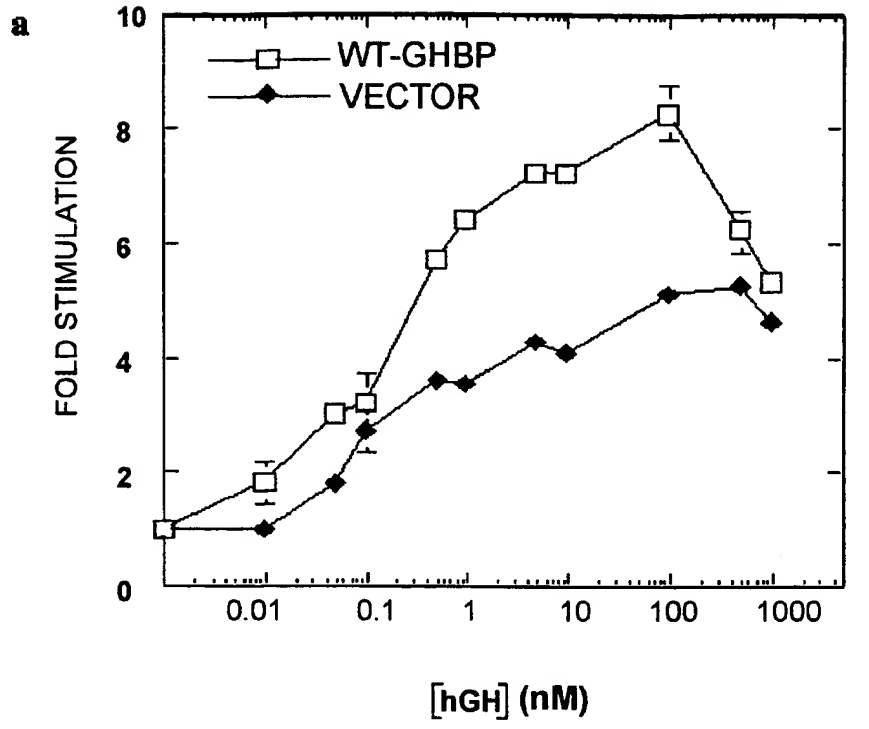
Fig. 2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1000
[hGH] (nM)

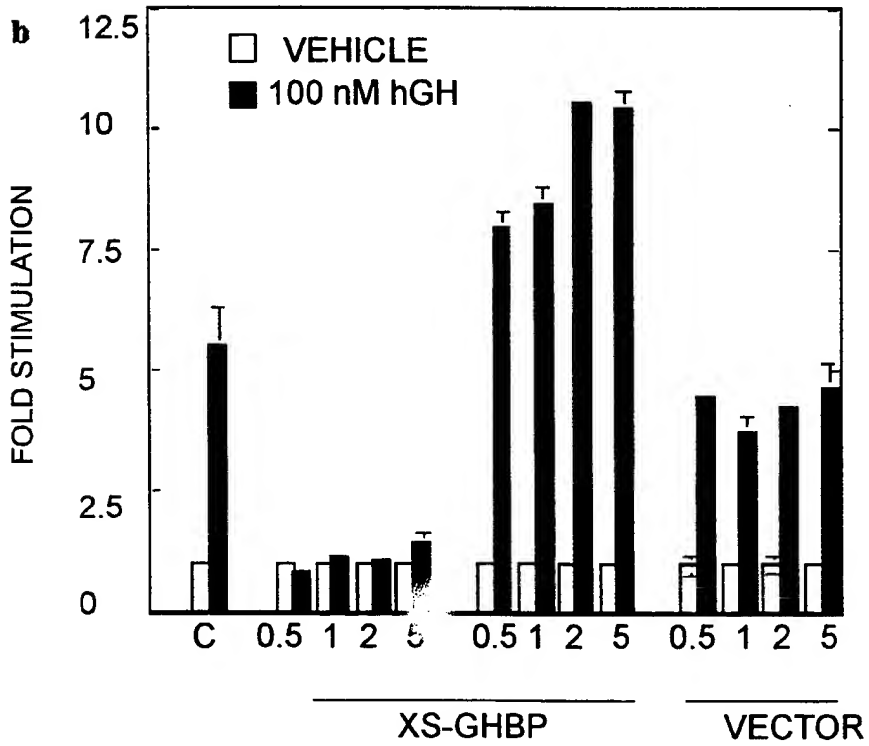
b
VEHICLE
100 nM hGH
FOLD STIMULATION
0
2.5
5
7.5
10
12.5
C
0.5
1
2
5
0.5
1
2
5
0.5
1
2
5
XS-GHBP
VECTOR
GH-RECEPTOR

Fig. 3

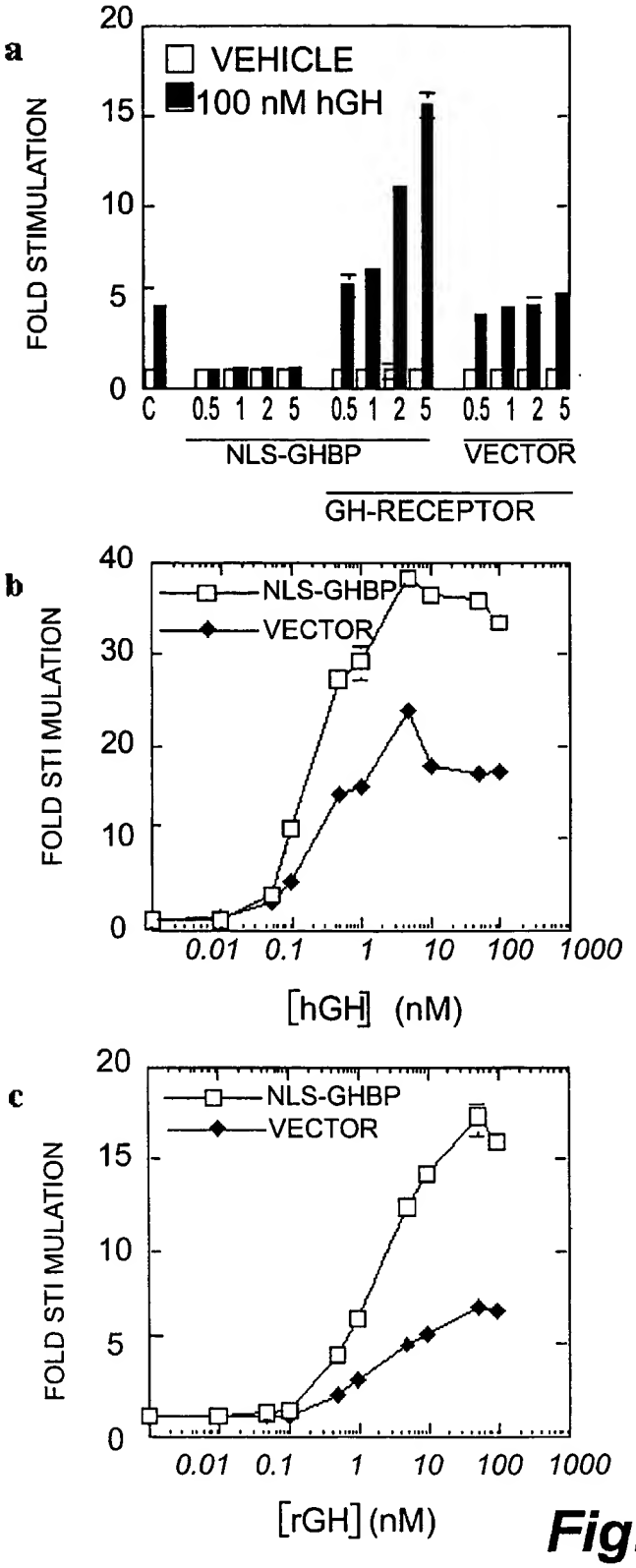
a
VEHICLE
100 nM hGH
FOLD STIMULATION
20
15
10
5
0
C
0.5 1 2 5
0.5 1 2 5
0.5 1 2 5
NLS-GHBP
VECTOR
GH-RECEPTOR
b
NLS-GHBP
VECTOR
FOLD STI MULATION
40
30
20
10
0
0.01 0.1 1 10 100 1000
[hGH] (nM)
c
NLS-GHBP
VECTOR
FOLD STI MULATION
20
15
10
5
0
0.01 0.1 1 10 100 1000
[rGH] (nM)

Fig. 4

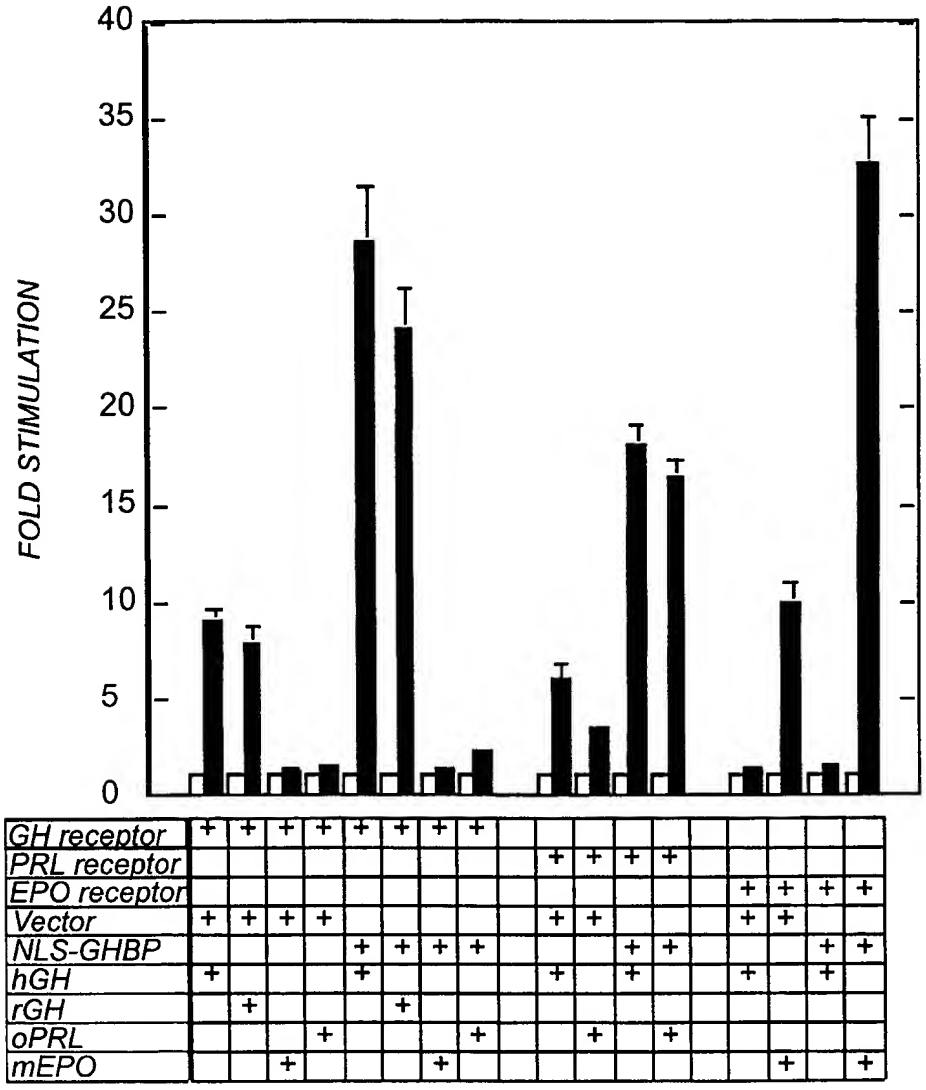
FOLD STIMULATION
40
35
30
25
20
15
10
5
0
GH receptor
PRL receptor
EPO receptor
Vector
NLS-GHBP
hGH
rGH
oPRL
mEPO

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/00478

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/62, A01K 67/027, A61K 0/00 // C07K 19/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, WPI, EPODOC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FASEB journal, Volume 8, No 1, August 1994, David A. Jans, "Nuclear signaling pathways for polypeptide ligands and their membrane receptors", page 841 - page 847, see especially page 844, column 1, lines 17-38	26-49
A	--	1-25
X	Bailliére's Clinical Endocrinology and Metabolism, Volume 10, No 3, July 1996, Marie-Catherine Postel-vinay et al, "Growth hormone receptor signalling", page 323 - page 336, see sections Janus kinases; STAT proteins; MAP kinases; GH-sensible genes; summary	26-49
A	--	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 June 1999	16 -07- 1999
Name and mailing address of the ISA/ Swedish Patent Office, Box 5055, S-102 42 STOCKHOLM, Facsimile No. +46 8 666 02 86	Authorized officer: Hampus Rystedt/EÖ, Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/00478

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The Journal of Biological Chemistry, Volume 269, No 50, December 1994, Peter E. Lobie et al, "Nuclear Translocation and Anchorage of the Growth Hormone Receptor", page 31735 - page 31746,see especially page 31745, lines 9-35 --	1-25
A	Growth Hormone and IGF Research, Volume 8, 1998, M.J. Thomas, "The molecular basis of growth hormone action" page 3 - page 11 --	1-49
A	Proceedings of the Society for experimental biology and medicine, Volume 206, No 3, 1994, Michael J. Waters et al, "Signal Transduction by the Growth Hormone Receptor" page 216 - page 220 --	1-49
A	The Journal of Biolocical Chemistry, Volume 273, No 9, 1998, Scott W. Rowlinson et al, "Activation of Chimeric and Full-length Growth Hormone Receptors by Growth Hormone Receptor Monoclonal Antibodies" page 5307 - page 5314 -- --------	1-49

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 99/00478

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

The application relates to four separate inventions, namely:

1. A DNA-construct encoding Growth Hormone Binding Protein (GHBP) with an appended Nuclear Localisation Sequence (NLS-GHBP), and uses thereof according to claims 1-25.

2. Use of a low-molecular, plasma membrane permeable substance which stimulates interactions between GHBP and intracellular signalling molecules according to claims 26, 34, 42 (all completely), 29-33, 37-41 and 45-49 (all partially).

3. Use of a low-molecular, plasma membrane permeable substance which simulates GHBP in intracellular signalling according to claims 27, 35, 43 (all completely), 29-33, 37-41 and 45-49 (all partially).

4. Use of a low-molecular, plasma membrane permeable substance which enhances the intracellular production of GHBP, according to claims 28, 36, 44 (all completely), 29-33, 37-41 and 45-49 (all partially).

The general concept of the application is to enhance the intracellular signalling action of GHBP in order to enhance growth and/or production in cells, in order to study the JAK-STAT pathway or in order to treat various diseases.
The involvement of GHBP in the GH signalling pathway is previously known, as the applicant states in page 2 lines 20-33 of the description. However, GHBP as a *location dependent* (nuclear) transcriptional enhancer and the *ligand* (GH) *independent* involvement of GHBP in intracellular signalling are novel. The special technical feature characterizing NLS-GHBP, as described in the application page 6 lines 2-10, is the increased amount of GHBP that may participate in STAT5 mediated transcription in the nucleus. Claims 1-25 cover this feature. However, claims 26-28 are very broad (they cover both ligand dependent and ligand independent interactions between GHBP and all cytoplasmic and nuclear signalling molecules) and also very vague in that not even the description suggests any kind of substances that might work. Thus, claims 26-28 contain solutions to problems which partly cover the same technical area but solve problems also outside the scope of claim 1. No common general concept defining a contribution above the prior art exists in the claims 26, 27 and 28. Consequently, the claims do not fulfill the requirements of unity of invention according to PCT Rule 13.1.